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(54) Title: PROCESS FOR THE MANUFACTURE OF PROBIOTIC CHEESE

(57) Abstract

A process for the manufacture of a probiotic cheese, such as Cheddar cheese, comprises adding a 0.05–0.5 % inoculum of a strain of *Lactobacillus paracasei*, which is non–pathogenic, acid and bile tolerant and adherent to human epithelial cells, as a starter adjunct to cheese milk, said *L. paracasei* strain being capable of growing during the ripening phase to a level of 10^7cfu/g or greater. The *L. paracasei* strains are found to grow and proliferate to high cell numbers (in excess of 10^8cfu/g) in the cheese over eight months of ripening, even when added at a relatively low inoculum. The presence of the *L. paracasei* strains is found to have negligible effects on cheese composition, flavour and aroma.

Description

Process for the manufacture of probiotic cheese

Technical Field

This invention relates to the manufacture of probiotic cheese and, in particular, to the manufacture of a probiotic cheese which contains at the time of consumption a viable, actively growing strain of an added bacterium.

Background Art

The importance of probiotic-containing products to maintenance of health and well-being is becoming a key factor affecting consumer choice, resulting in rapid growth and expansion of the market for such products, in addition to increased commercial interest in exploiting their proposed health attributes. The majority of probiotic foods already on the market, such as fermented milks and yoghurt are fresh products and are generally consumed within days or weeks of manufacture. In contrast, hard cheeses, such as Cheddar have long ripening times of up to two years.

Probiotic bacteria are described as 'living' micro-organisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition. Probiotics may be consumed either as a food component or as a non-food preparation. Foods containing such bacteria fall within the 'functional foods' category and these are described as 'foods claimed to have a positive effect on health'. Such products are gaining more widespread popularity and acceptance throughout the developed world and are already well accepted in Japan and the USA. Furthermore, increased commercial interest in exploiting the proposed health attributes of probiotics has contributed in a significant way to the rapid growth and expansion of this sector of the market.

The potential health-promoting effects of dairy products which incorporate probiotic organisms such as *Lactobacillus* and *Bifdobacterium* spp. has stimulated a major research effort in recent years. To date, the most popular food delivery systems for these cultures have been freshly fermented dairy foods, such as yoghurts and fermented milks, as well as unfermented milks with cultures added.

There are relatively few reports concerning cheese as a carrier of probiotic organisms, even though there are a small number of 'probiotic cheeses' currently on the market.

In 1994, Dinakar and Mistry (J. Dairy Sci. *77:2854-2864)* incorporated *Bifidobacterium bifidum* into Cheddar cheese as a starter adjunct. This strain survived well in the cheese and retained a viability of approximately 2 x 10^7 cfu/g even after 6 months of ripening, without adversely affecting cheese flavour, texture or appearance. This example suggested that Cheddar could provide a suitable environment for the maintenance of probiotic organisms at high levels over long time periods. However, no growth of the *B. bifidum* was observed in the cheese during the ripening period and thus it is important to emphasise that the Bifidobacterium strain did not grow during manufacture and/or ripening and thus had to be added at a relatively high inoculum. In another study, bifidobacteria were used in combination with *Lb. acidophilus* strain Ki as a starter in Gouda cheese manufacture (Gomes, A.M.P. *et al* (1995); Neth. Milk Dairy J. *49*:71-95). The two strains were used as sole starters, requiring relatively large inocula (3%) of both strains and adaptation of cheese making technology. In this case, there was a significant effect on cheese flavour in the resultant product after 9 weeks of ripening, possibly due to acetic acid production by the bifidobacteria.

In order to exert a probiotic effect, cultures must maintain their viability in food products through to the time of consumption, which for Cheddar cheese is many months after manufacture.

Cheese is a milk product in which the whey protein/casein ratio does not exceed that of milk and which is obtained by coagulation of milk by the action of rennet, followed by whey drainage. Starter cultures containing lactic acid bacteria are initially required during cheese making to metabolise lactose, thereby producing lactic acid and reducing the pH. During Cheddar cheese manufacture for example, the starter lactococci grow, reaching maximum levels of approximately 10^9 to 10^{10} cfu/g at salting. Conditions in the cheese however, such as high salt in moisture (S/M), low pH, lack of a fermentable carbohydrate and low temperature of ripening can result in a dramatic decline in starter numbers during the early weeks of ripening. The rate of decline depends on a number of characteristics of the strain, including autolytic properties, salt tolerance and phage resistance. In the meantime, a population of non-pathogenic organisms, referred to as non-starter-lactic acid bacteria (NSLAB), chiefly composed of lactobacilli (*Lb. plantarum, casei* and *brevis*) and pediococci (*Pediococcus pentosaceus*) proliferate as the cheese ripens, a process that is generally performed at 2-16°C. It is believed that NSLAB gain access to the cheesemilk during the manufacturing stage or that they survive pasteurisation in an attenuated state. Regardless, their numbers increase rapidly reaching maximum levels of 10^7 to 10^8 cfu/g in ripened Cheddar cheese. Indeed, in mature cheese, NSLAB may represent the principal flora. Their role in determining cheese quality remains unclear. NSLAB are generally enumerated using an aerobic plate count on Rogosa or Lactobacillus Selective (LBS) agar.

It may not be cost-effective to add probiotic strains to cheese in amounts corresponding to that finally required for a probiotic product at time of consumption. Rather what is required is a probiotic strain which can be added as a starter adjunct at a low inoculum to cheese and which grows to the required values of $\sim >10^7$ cfu/g.

What is required, therefore, for a probiotic cheese with a long ripening time such as Cheddar is a probiotic strain which can survive and grow throughout manufacture and the ripening period.

Disclosure of Invention

The invention provides a process for the manufacture of a probiotic cheese, which process comprises adding a 0.05-0.5% inoculum of a strain of *Lactobacillus paracasei*, which is non-pathogenic, acid and bile tolerant and adherent to human epithelial cells, as a starter adjunct to cheese milk, said *L. paracasei* strain being capable of growing during the ripening phase to a level of 10^7cfu/g or greater.

We have found that said strain of *L. paracasei* has the ability to survive the cheese manufacturing process and the capacity to grow and survive during the ripening/storage period. The strain of *L. paracasei* used in the process according to the invention also has the ability to survive passage through the gastrointestinal tract as hereinafter demonstrated. The presence of the added *L. paracasei* strain has been found to have negligible effects on cheese composition, flavour and aroma.

Preferably, a 0.1-0.25% inoculum of the *L. paracasei* is added to the cheese milk.

Also, preferably the ripening phase is at least six months.

Further, preferably, the ripening phase is eight months or greater.

The *L. paracasei* strains used in the process according to the invention have been found to grow and proliferate to high cell numbers in cheese over eight months of ripening, when added at a low inoculum as described herein.

Thus, in one embodiment of the invention, the *L. paracasei* is capable of growing during the ripening phase to a level of 10^8cfu/g or greater.

Preferably, the *L. paracasei* is tolerant to temperatures of 37°C or greater.

Also, preferably the *L. paracasei* can be enumerated and distinguished from the resident flora.

Most preferably, the added *L. paracasei* cells are enumerated and distinguished by a randomly amplified polymorphic DNA (RAPD) method which allows the generation of discrete DNA fingerprints for the respective strains.

The RAPD used allowed the generation of discrete DNA fingerprints for each strain which were clearly distinguishable from those generated by the natural flora of the cheeses.

Preferably, the cheese manufactured is a hard cheese.

In an especially preferred embodiment the cheese is Cheddar cheese.

Cheddar cheese has particular advantages as a carrier of a probiotic micro-organism as described herein. Having a higher pH than the more traditional probiotic foods (e.g. yoghurts and fermented milks), it provides a more stable milieu to support their long-term survival. Furthermore, the matrix of the cheese and its relatively high fat content offers protection to probiotic bacteria during passage through the gastrointestinal tract (GIT).

The *L. paracasei* strains used in accordance with the invention were obtained from University College Cork, under a restricted Materials Transfer Agreement, together with a number of other strains for the purposes of investigation as described in the Examples.

The *L. paracasei* strains were found to have the requisite properties for use in cheese manufacture whereas, for example, the

Lactobacillus salivarius strains investigated died during the ripening period.

The *L. paracasei* strains used herein have the requisite ability to influence the microflora of both the cheese and the GIT, the ability of the culture to grow in dairy-based media, such as whey and phage inhibitory media and the ability of the culture to survive and/or grow during manufacture and throughout the shelf-life of the cheese product.

The invention also provides *Lactobacillus paracasei* strain NFBC 338 or a mutant or variant thereof.

Also the invention provides *Lactobacillus paracasei* strain NFBC 364 or a mutant or variant thereof.

Samples of these bacteria have been deposited at The National Collections of Industrial and Marine Bacteria Limited (NCIMB) on May 29, 1998 and have been accorded the accession numbers NCIMB 40954 and NCIMB 40955, respectively.

In a further embodiment of the invention there is provided a probiotic cheese ready for consumption which contains a viable, actively growing strain of *L. paracasei* as hereinbefore defined in an amount of 10^7cfu/g or greater, following manufacture thereof using said *L. paracasei* as a starter adjunct.

An especially preferred cheese is Cheddar cheese.

Probiotic Cheddar cheeses can be manufactured in accordance with the invention containing high levels of *L. paracasei* strains (10^8 cfu/g cheese) at a relatively low cost to the producer and using identical manufacturing procedures. Importantly, we have shown that incorporation of these strains does not impact negatively on cheese quality, including aroma, flavour and texture. In addition, our results suggest that cheese also compares very favourably with yoghurt

regarding delivery of viable cells to the GIT despite the apparent age difference of the products.

Brief Description of Drawings

Fig. 1A is a graph of log cfu/g *versus* time (days) representing survival of lactobacilli and starter during cheese ripening in Trial 1 as described in Example 2;

Figs. 1B-1D are RAPD PCR profiles of a representative number of *Lactobacillus* isolates from each of the cheeses as described in Example 2;

Fig. 2A is a graph of log cfu/g *versus* time (days) representing survival of lactobacilli and starter during cheese ripening in Trial 2 as described in Example 2;

Figs. 2B-2E are RAPD PCR profiles of a representative number of *Lactobacillus* isolates from each of the cheeses as described in Example 2;

Fig. 3A is a graph of log cfu/g *versus* time (days) representing survival of Lactobacilli and starter during cheese ripening in Trial 3 as described in Example 2;

Fig. 3B depicts RAPD PCR profiles of a representative number of *Lactobacillus* isolates from Vat 2 cheese as described in Example 2;

Fig. 4A is a graph of log cfu/g *versus* time (days) representing survival of Lactobacilli and starter during cheese ripening in Trial 4 as described in Example 2;

Fig. 4B depicts RAPD PCR profiles of a representative number of Lactobacillus isolates from Vat 2 cheese as described in Example 2

Fig. 5 is a urea PAGE of control and experimental Cheddar cheeses after eight months of ripening as described in Example 5;

Fig. 6A shows the concentration of individual free amino acids in water-soluble extracts of six month old control and experimental cheeses found in Trial 1 as described in Example 5; and

Fig. 6B shows the concentration of individual free amino acids in water-soluble extracts of six month old control and experimental cheeses found in Trial 2 as described in Example 5.

Modes for Carrying Out the Invention

The invention will be further illustrated by the following examples:

Example 1

Probiotic strain identification/enumeration

A pre-requisite to the successful enumeration of added probiotic strains is to be capable of selectively identifying these from the natural, often complex microflora found in food products. Since NSLAB can reach levels of up to 10^7 - 10^8 cfu/g in cheese during ripening it was necessary to evaluate a number of methods aimed at selectively enumerating the lactobacilli added as starter adjuncts from these NSLAB.

The probiotic *Lactobacillus* strains used in this Example had previously been isolated from the human gastrointestinal tract, and were obtained from Prof. J.K. Collins, Microbiology Dept., University College Cork, Ireland under the aforementioned Materials Transfer Agreement. These strains were identified as *L. salivarius* (ssp. *salivarius*) and *L. paracasei* (ssp. *paracasei*) by SDS-PAGE analysis of total cell protein. (Reuter, G. (1990) Bifidobacteria microflora 9:107-118) and were designated *Lb. salivarius* NFBC 310, NFBC 321 and

NFBC 348 and *L. paracasi* NFBC 338 and NFBC 364. NSLAB *Lactobacillus* strains (*Lb. curvatus* DPC 2042 and 2081, *L. plantarum* DPC 2102 and 2142 and *L. casei* ssp. *casei* DPC 2047 and 2103) which had previously been isolated from 8 week old commercial Cheddar cheeses, were obtained from the culture collection of the Dairy Products Research Centre. All *Lactobacillus* strains were routinely cultured in MRS broth (Dinakar, P. and V.V. Mistry (1994)) (Difco Laboratories, Detroit, MI, USA) under anaerobic conditions (anaerobic jars with 'Anaerocult A' gas packs; Merck, Darmstadt, Germany) at 30°C and 37°C for NSLAB and probiotic strains, respectively. Solid media were prepared by adding 1.5% agar to broth medium. Stock cultures were maintained at -80°C in 40% glycerol-supplemented MRS broth. Each culture was sub-cultured twice in MRS broth before use from stock. *Lactococcus lactis* ssp. *cremoris* strains 227 and 223, obtained from Chr. Hansen's Laboratories (Little Island, Cork, Ireland) in the form of freeze-dried pellets, were used as starters for cheese-making. These were grown overnight at 21°C in heat-treated (90°C for 30 min) 10 % (w/v) reconstituted skim-milk (RSM).

<u>Bacteriological analyses of cheeses</u>

Viability of lactobacilli (both probiotic adjuncts and NSLAB) in the inoculated cheese-milk and in the cheeses during ripening was determined on LBS agar after 5 days of anaerobic incubation at 30°C while starter lactococci were enumerated on LM17 agar after 3 days incubation at 30°C. Coliforms were enumerated in cheese-milk and cheeses on Violet Red Bile Agar (VRBA; Oxoid) at 37°C for 24 hours. Cheeses were aseptically sampled in duplicate for bacteriological analysis, at intervals during ripening. Cheese samples were emulsified in sterile 2 % (w/v) trisodium citrate, diluted in maximum recovery diluent and appropriate dilutions pour-plated. After 1, 3 and 6 monthly intervals, 20 individual *Lactobacillus* colonies from each cheese were randomly selected from the LBS agar plates for RAPD-PCR analysis.

a) bile and temperature tolerance of *Lactobacillus* adjuncts

To investigate the tolerance of both the probiotic and NSLAB *Lactobacillus* isolates to bile, overnight MRS broth cultures of each of the *Lactobacillus* strains were serially diluted in maximum recovery diluent (Oxoid Ltd, Basingstoke, Hampshire, UK) and appropriate dilutions pour-plated on MRS agar with 0, 0.1, 0.3, 0.5, 1.0 or 3.0 % porcine bile (Sigma Chemical Co., Poole, Dorset, England). After 3 days incubation, the plates were examined and where colonies were present, their numbers and sizes were recorded. Temperature tolerance of the probiotic lactobacilli was investigated by pour-plating appropriate dilutions of overnight cultures on LBS agar (Rogosa, M. *et al.* (1951) J. Bateriol *62*:132-133) (Becton Dickinson, Cockeysville, MD, USA) and incubating the plates anaerobically both at 37 °C (which is the optimum temperature of growth for these strains) and at 42 °C. Colony numbers obtained after 5 days were compared. In the same way, the temperature tolerance of these strains and NSLAB, following isolation from Cheddar cheese was also investigated.

The bile and temperature tolerance of both the human-derived lactobacilli and a selection of NSLAB was first determined in the hope that either of these parameters could form a basis for the selection of the adjunct from the product. Both the probiotic adjuncts and NSLAB *Lactobacillus* strains varied considerably with regard to their bile tolerance. Two of the NSLAB isolates used in this Example were tolerant to levels of porcine bile of up to 3% compared to the lactobacilli added as starter adjuncts, which were inhibited at 0.3% bile. Therefore selections based on bile tolerance would not be useful in distinguishing the probiotic adjunct lactobacilli incorporated into Cheddar cheese in this Example from the NSLAB lactobacilli. Similarly, temperature tolerance could not be used as a basis for selection of the probiotic lactobacilli from NSLAB. NSLAB isolated from Irish Cheddar cheeses do not grow at 45°C while some of the human-derived probiotic lactobacilli may withstand such temperatures (Kandler, O. and Weiss, N. (1989) *In* P.H. A. Sneath (ed.), Bergey's manual of determinative bacteriology, Vol. 2. The Williams & Wilkins

Co., Baltimore, Md.). A temperature of 42°C was evaluated for selective enumeration of the probiotic strains from the NSLAB. While the probiotic *Lactobacillus* strains, isolated from fresh cultures or Cheddar cheese early in ripening were capable of growth at 42°C, they failed to grow at this temperature when isolated from mature cheese. Furthermore, some NSLAB lactobacilli were found to be capable of growth at 42°C, confirming that this procedure was non-selective for the human-derived probiotic *Lactobacillus* strains from Cheddar cheese.

b) <u>RAPD-PCR analysis</u>

RAPD-PCR analysis was carried out on each of the probiotic *Lactobacillus* strains and on cultures grown from *Lactobacillus* colonies isolated from Cheddar cheese. Genomic DNA was isolated from 1.5 ml of overnight MRS broth cultures using a modification of the method of Hoffman and Winston (Hoffman, C.S., and Winston, F. (1997) Gene *57*:267-272). This procedure utilises shearing with glass beads to lyse the cells, and was modified as outlined by Coakley *et al.* (Coakley,M. *et al.* (1996); J. Inst. Brew. *102*:344-354). One microlitre of the extracted DNA was used in subsequent PCR amplifications, which were performed in a total volume of 25 µl in a Perkin-Elmer (Norwalk, CT, USA) DNA Thermal Cycler. The method employed was essentially as described by Coakley *et al.* ((1996) *supra*) and used a single primer of arbitrary nucleotide sequence (5' ATGTAACGCC 3'), obtained from Pharmacia Biotech, (Uppsala, Sweden). DNA was amplified for 35 cycles using the following temperature profile: denaturing at 93 °C for 1 min, annealing at 36 °C for 1 min followed by polymerisation at 72 °C for 1 min. *Taq* DNA polymerase (0.625 Units, Bioline) was added to the reaction mix during the first denaturation step (Hot Start). Between 5 and 10 µl of the PCR reaction was analysed on a 1.5 % (w/v) agarose (Sigma) gel with ethidium bromide staining. A 100 bp ladder (Pharmacia) was used as a molecular weight standard. Gels were run for approximately 3 hours at 100 V and the DNA visualised by UV transillumination.

Consequently, the Randomly Amplified Polymorphic DNA (RAPD) method, which involves PCR using an arbitrary primer, was used to generate DNA fingerprints for each of the probiotic strains. Each of the *Lactobacillus* strains generated reproducible discrete DNA fingerprints, which were found to be substantially different from those of representative NSLAB lactobacilli. Thus, the RAPD method proved to be a successful means of identifying the probiotic strains and demonstrated potential as a means of selective identification of the strains from the NSLAB flora in Cheddar cheese.

Example 2

Incorporation of *Lactobacillus* species into Cheddar cheese

Laboratory-scale cheesemaking trials (Trials 1 and 2) were performed initially using 25 L of pasteurised whole milk in each cheese vat. To limit contamination with wild lactobacilli, these cheeses were manufactured under controlled bacteriological conditions, as described by McSweeney, P. *et al.* ((1994); Irish J. Agric. Food Res. *33*:183-192). A 1.5 % inoculum of the mixed-strain starter culture was used and in each trial one vat (Vat 1) acted as a control to which starter only was added. To each of the experimental vats, one probiotic *Lactobacillus* strain, grown overnight in 10 % RSM, was added as an adjunct to the starter culture. In Trial 1, the probiotic adjuncts *L. salivarius* NFBC 348 and *L. paracasei* NFBC 364 were added at an inoculum level of 0.1 % to Vats 2 and 3, respectively. In the second trial, *L. salivarius* NFBC 310 (Vat 2), *L. salivarius* NFBC 321 (Vat 3) and *L. paracasei* NFBC 338 (Vat 4) were inoculated at a level of 0.2 %. Cheddar cheeses were then manufactured according to standard procedures as follows: Filter-sterilised rennet (Chr. Hansen's Laboratories) was added at a concentration of 0.07 ml/liter 35 min after starter and adjunct addition, and the curd was cut approximately 40 min later. Curds were cooked to 39 °C, pitched at pH 6.1 and milled at approximately pH 5.3. Salt was added at a rate of 2.8 % (w/w) and the curds were placed in moulds and pressed at approximately 200 kPa overnight. The cheeses were removed from the moulds, vacuum-

packed and ripened at 8 °C for approximately 8.5 months. Subsequently two pilot-scale cheesemaking trials (Trials 3 and 4) were performed using two of the adjunct *Lactobacillus* strains which were found to maintain high viability in the laboratory-scale cheeses during ripening. In each trial, two vats, one experimental and one control, each containing 450 liters of standardised (fat:protein = 1) pasteurised whole milk were used. As in the laboratory-scale trials, a 1.5 % inoculum of the starters 223 and 227 was added to each vat. In addition, in each trial the experimental vat (Vat 2) contained a 0.1 % inoculum of either *L. paracasei* NFBC 364 (Trial 3) or NFBC 338 (Trial 4) added as a starter adjunct. The cheesemaking procedure was as previously described for the laboratory-scale cheeses except that the salting level was 2.7 % and the curds were pressed overnight at approximately 413 kPa.

Initially, laboratory-scale cheese trials were performed under microbiologically controlled conditions (thus limiting development of high numbers of NSLAB during ripening) to assess the performance of five probiotic *Lactobacillus* strains in Cheddar cheese. Firstly, for inoculation purposes, the performance of these strains in RSM was investigated. None of the strains performed well in milk (levels of only 10^7 - 10^8 cfu/ml achieved) and were subsequently found to be non- or only weakly proteolytic (data not shown). Thus, using a 0.1 - 0.2 % inoculum of these *L. salivarius* and *paracasei* strains as starter adjuncts, relatively low levels of 10^4 - 10^5 cfu/ml milk were obtained in the experimental vats during cheese manufacture as shown in Table 1. All adjunct lactobacilli were found to survive the cheese manufacturing process and, given their poor growth in milk and the low inoculum used, were shown to have no effect on acid production during the process (data not shown). Results demonstrate that cheese made with NFBC 364 and NFBC 338 *L. paracasei* adjuncts (Trial 1 Vat 3, Trial 2, Vat 4, respectively) contained high levels of these probiotic strains after 8 months of ripening; with final counts of 9.2 x 10^7 and 1.4 x 10^8 cfu/g achieved, respectively as shown in Figs. 1A and 2A.

Table 1

Baterial counts (cfu/ml) in milk used for the manufacture of Cheddar cheese, after inoculation with adjunct and/or starter cultures

Cheese inoculum[1]	Lactobacilli	Lactococci
Trial 1[3]		
V1, 1.5% 227/223	ND[2]	3.2×10^6
V2, 1.5% 227/223 + 0.1% *L. salivarius* NFBC 348	1.3×10^5	3×10^6
V3, 1.5% 227/223 + 0.1% *L. paracasei* NFBC 364	2.4×10^5	2.9×10^6
Trial 2[3]		
V1, 1.5% 227/223	ND	2.7×10^6
V2, 1.5% 227/223 + 0.2% *L. salivarius* NFBC 310	2.9×10^5	3.8×10^6
V3, 1.5% 227/223 + 0.2% *b. salivarius* NFBC 321	2×10^5	2.8×10^6
V4, 1.5% 227/223 + 0.2% *L. paracasei* NFBC 338	2.3×10^4	4.5×10^5
Trial 3[4]		
V1, 1.5% 227/223	ND	2.4×10^6
V2, 1.5% 227/223 + 0.1% *L. paracasei* NFBC 338	1.7×10^5	4.1×10^6
Trial 4[4]		
V1, 1.5% 227/223	ND	3.10^5
V2, 1.5% 227/223 + 0.1% *L. paracasei* NFBC 364	8.9×10^5	1.1×10^6

[1]227/223 = *L. Lactis* ssp. *cremoris* 227+223

[2]ND = Non-detectable

[3]Trial 1 and 2 cheeses manufactured at laboratory-scale under microbiologically controlled conditions

[4]Trial 3 and 4 cheeses manufactured at pilot-scale using the two *Lactobacillus* adjunct strains (NFBC 338 and NFBC 364) which showed good survival in the laboratory-scale cheeses during ripening

Figs. 1B-1D are RAPD PCR profiles of a representative number of *Lactobacillus* isolates from each of the cheeses (B, C and D); Lane 1 shows the RAPD profile of the probiotic *Lactobacillus* strain added to the cheese at manufacture, while a 100 bp ladder is shown at Lane 19 (B) and Lane 11 (C and D) and all other lanes (B, C and D) show RAPD profiles of *Lactobacillus* isolates from 6-month-ripened cheeses.

Figs. 2B-2D are RAPD PCR profiles of a representative number of *Lactobacillus* isolates from each of the cheeses (B, C, D and E); Lane 1 shows the RAPD profile of the probiotic *Lactobacillus* strain added to the cheese at manufacture, while a 100 bp ladder is shown in Lane 19 (B, C, D and E) and all other lanes (B, C, D, and E) show RAPD profiles of *Lactobacillus* isolates from 6-month-ripened cheeses.

The high levels of probiotic strains was confirmed following comparison of the RAPD PCR fingerprints generated for *L. paracasei* strains NFBC 364 and NFBC 338 (Fig. 1D and Fig. 2E, lane 1) and those obtained for lactobacilli isolated from the cheeses (Fig. 1D and Fig. 2E, lanes 2-10 and 12-20) which were found to be identical. In contrast, although lactobacilli grew to high levels (1 x 10^8 cfu/g) in the cheese to which strain NFBC 310 was added (Trial 2 Vat 2), and subsequently remained at this level throughout ripening (Fig. 2A), these lactobacilli (Fig. 2C, lanes 2-10 and 12-2-) were identified by RAPD PCR as NSLAB. Levels of lactobacilli in cheeses with *L. salivarius* adjuncts NFBC 348 and NFBC 321 (Trial 1 Vat 2 and Trial 2 Vat 3, respectively) declined to 1.2 x 10^5 cfu/g and 8.6 x 10^4, respectively, after 4 months of ripening (Figs. 1A and 2A), although these levels did increase slightly to reach final levels of 3.5 x 10^5 and 1.1 x 10^6 cfu/g, respectively after 8 months of ripening. Interestingly, the genetic fingerprints of isolates taken from each of these cheeses after 6 months revealed that these lactobacilli were predominantly NSLAB (Fig. 1C and Fig. 2D, respectively). Thus, the *L. salivarius* strains used in this Example did not maintain viability in Cheddar cheeses during ripening. Furthermore, many of the NSLAB isolated from these cheeses in which the adjunct strains declined (Fig. 1C, lanes 3-6 and Fig. 3D, lanes 12-18) and from the control cheeses to which no probiotic adjuncts were

added (Fig. 1B, lanes 9-13 and Fig. 2B, lanes 3-9) yielded identical PCR-generated DNA fingerprints. This suggests that the DNA was obtained from identical strains and shows a predominance of certain *Lactobacillus* strains in the NSLAB population of these cheeses.

Subsequently, pilot-scale cheese trials were performed, where only the two *L. paracasei* strains, NFBC 338 and NFBC 364, which survived to high levels in the laboratory-scale trials were incorporated into Cheddar cheese. These strains were added to Trial 3 Vat 2 (NFBC 338) and Trial 4 Vat 2 (NFBC 364) at inocula of 1.7 and 8.9 x 10^5 cfu/ml cheese-milk, respectively as shown in Table 1. Thereafter, both NFBC 338 and NFBC 364 grew in the cheese from initial numbers of 1.1. x 10^7 and 2.7 x 10^7 cfu/g, respectively, to reach levels of between 1.5 and 2.9 x 10^8 cfu/g after 3 months of ripening and viability was sustained at this level for the remainder of the ripening period (Figs. 3A and 4A). As in the laboratory-scale cheeses, these results were confirmed by RAPD PCR analysis (as described in Example 1) of a number of isolates from each of these cheeses (Figs. 3B and 4B).

Taken together, the data from the laboratory- and pilot-scale cheese trials provide molecular-based evidence for the persistence in Cheddar cheese of strains selected for their potential as probiotics. In order to appreciate the beneficial effects of 'probiotic' foods, it has been proposed as indicated above, that viable probiotic organisms should be present at levels of at least 10^7 viable cells per gram or millilitre of product. The probiotic-containing cheeses obtained in accordance with the invention contained levels of up to 10^8 cfu/g cheese, thus satisfying the criteria for a 'probiotic' food product.

It should also be noted that lactococcal starter numbers in the control cheeses of all trials showed a typical decline during the ripening period (Figs. 1A, 2A, 3A and 4A). However, due to the growth of lactobacilli on the LM17 medium used to enumerate these starter organisms, it was possible only to monitor starter in these cheeses, to which no adjunct lactobacilli had been added, and then only in the early stages of ripening.

RAPD PCR analysis, when used as an identification method, was capable of determining that probiotic *L. paracasei* strains grew and maintained high viability (10^8 cfu/g) in cheese, while the particular *L. salivarius* adjunct strains used did not appear to be suited for such an application. Furthermore, survival of these probiotic *Lactobacillus* strains at high numbers in Cheddar cheese was achieved using a relatively low inoculum (0.1 - 0.2%) in the cheese vat and without altering the cheesemaking process in any way. This was possible because these strains were added as starter adjuncts and were not therefore necessary for acid production during cheese-making. Thus, the process according to the invention for incorporation of probiotic organisms into Cheddar cheese offers certain advantages to industry; no alteration of existing cheese-making technology and low cost due to the low inoculum required.

Example 3

Cheese compositional analysis

Grated cheese samples were analysed in duplicate for salt by a potentiometric method (Irish Dairy Federation (1979); Cheese and processed cheese. Determination of chloride content: potentiometric titration method. IDF Standard 88), fat by the Gerber method (Irish Standard (1955); Determination of the percentage fat in cheese. Irish Standard. 69), moisture by oven-drying at 102°C (Irish Dairy Federation (1982); Determination of the total solids content (cheese and processed cheese). IDF Standard 4A) and protein on a LECO FP-428 nitrogen determinator. The pH of a slurry, prepared by blending 12 ml H_2O with 20 g grated cheese, was measured using a standard pH meter (Radiometer, Copenhagen, Denmark).

The composition of the cheese was generally found to be within the range typical for Cheddar as shown in Table 2.

Table 2

Composition[1] of control and probiotic Cheddar cheeses

Cheese trial	Moisture	Salt	S/M[2]	Fat	Protein	pH
			(%)			
Trial 1						
V1	38.28	1.53	4.0	31.5	26.33	5.4
V2	38.24	1.70	4.45	32.0	26.63	5.2
V3	39.89	1.23	3.08	31.0	25.79	5.3
Trial 2						
V1	37.48	1.64	4.38	33.0	26.5	5.2
V2	35.73	1.81	5.07	33.0	26.99	5.1
V3	37.22	1.61	4.33	33.0	27.27	5.1
V4	38.01	1.71	4.55	33.0	27.27	5.1
Trial 3						
V1	35.61	1.76	4.94	33	26.33	5.2
V2	36.74	1.72	4.68	33	26.56	5.2
Trial 4						
V1	34.88	2.05	5.88	34.5	26.17	5.4
V2	35.14	1.80	5.12	35.0	26.42	5.3

[1]Means of duplicate analyses

[2]Salt-in-moisture

Some atypical values for salt-in-moisture (Vat 3), fat (all vats) and pH (Vat 1) were obtained for the Trial 1 cheeses which reflects the difficulty in controlling the cheesemaking parameters (i.e. temperature) at a laboratory-scale. In contrast, all the compositional analysis values obtained for the pilot-scale trials were generally within the typical range for Cheddar. Thus, the comparable values observed for control and experimental cheeses (Table 2) indicate that incorporation of probiotic lactobacilli as starter adjuncts, and their survival at high numbers, had no direct effect on cheese composition.

Example 4

Sensory evaluation of Cheddar cheese

Cheeses were graded blindly after 3 and 6 months ripening by a commercial grader from a local cheese manufacturing plant. The cheeses were graded for flavour/aroma and body/texture, with maximum scores of 45 and 40, respectively. Minimum scores of 38 and 31 for flavour/aroma and body/texture, respectively are required for commercial Cheddar cheese. With the exception of the control cheese of Trial 2, all cheeses could be described as commercial grade with respect to sensory criteria, after 6 months of ripening, having achieved minimum scores of 38 and 31 for flavour/aroma and body/texture, respectively as shown in Table 3.

Table 3

Sensory evaluation of Cheddar cheeses at 6 months

Cheese	Flavour/aroma[1]	Body/texture[2]
Trial 1		
V1	38	33
V2	38	33
V3	39	32
Trial 2		
V1	37	33
V2	39	32
V3	38	33
V4	38	32
Trial 3		
V1	38	33
V2	38	33
Trial 4		
V1	39	33
V2	38	33

[1]Maximum score = 45; minimum commerical score = 38

[2]Maximum score = 40; minimum commercial score = 31

Lactobacillus adjuncts have previously been reported to improve Cheddar cheese flavour (Broome, M.D., *et al.* (1990); Aust. J. Dairy Technol. *45*:67-73) although, in some cases they were responsible for flavour defects (Puchades, R., *et al.* (1989); J. Food Sci. *54*:885-888). In this Example, laboratory-scale cheeses with high levels of *Lactobacillus* adjuncts were found to have flavour and texture comparable to that of control cheeses, indicating that addition of these probiotic lactobacilli to Cheddar cheese had no adverse effects on sensory criteria. Furthermore, when repeated on a larger scale, sensory parameters remained unaffected by the presence of high levels of these adjuncts.

Example 5

Proteolysis in laboratory-scale Cheddar cheeses

Cheeses were analysed by urea- PAGE (Shalabi, S.L., and Fox, P.F. (1987); Irish J. Food Sci. Technol. *11*:135-151) using a Protean II xi vertical slab gel unit (Bio-Rad Laboratories, Ltd, Watford, Herts, UK) essentially with the stacking gel system of Andrews (Andrews, A.T. (1983); J. Dairy Res. *50*:45-55). Cheese samples were prepared by dispersing 10 mg of grated cheese in 1 ml of sample buffer and heating at 50°C for 5 min. Samples were stored at -20°C until use and 10μl was applied to the gel. Sodium caseinate (5μl) was used as a standard for comparative purposes. Samples were electrophoresed at 280 V through the stacking gel and at 300 V through the resolving gel. Gels were stained with Coomassie Brilliant Blue G250 using the direct-staining procedure of Blakesley and Boezi (Blakesley, R.W:, and J. A. Boezi (1977); Anal. Biochem *82*:582-581).

Water-soluble extracts (pH 4.6) of each of the cheeses were prepared according to the method of Kuchroo and Fox (Kuchroo, C.N. and Fox, P.F.; (1982); Milchwissenshcaft *37*:331-335) and freeze-dried. The size distribution of peptides in these freeze-dried extracts was determined by size-exclusion HPLC, using a TSK 2000 SW (Beckman Instruments Ltd, High Wickham, United Kingdom) gel permeation

column (7.5nm x 60cm) fitted to a Waters HPLC system (Waters Chromatography Division, Milford, MA, USA). The column was eluted at a flow-rate of 1ml/min with 30% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The freeze-dried water-soluble extracts were reconstituted (3mg/ml) in HPLC-grade water, filtered through a Whatman 0.2μm filter and 20μl applied to the column. Column eluates were continually monitored at 214nm. Data were collected using a PC Minichrom system (VG Data Systems, Cheshire, United Kingdom) and results compared to a previously prepared calibration curve.

Individual free amino acids (FAA) in the water-soluble extracts were determined using a Beckman System 6300 High Performance Analyser (Beckman Instruments Ltd, High Wickham, United Kingdom) equipped with a Beckman P-N 338052 Na^+ column (12cm x 0.5cm) as described by Lynch *et al.*(Lynch, C.M. *et al.* (1996); Int. Dairy J. 6:851-867). Chromatograms were collected using a computer-controlled Minichrom data processing package. Amino acid concentrations were expressed as μg/ml cheese extract which were subsequently converted to μg/gcheese.

Urea-PAGE electrophoresis patterns of whole cheese samples after 8 months of ripening (Fig. 5) are typical for Cheddar and do not show any differences in the extent of primary proteolysis between the control cheeses and those manufactured with adjunct lactobacilli.

Fig. 5 represents Urea-PAGE of control (Lanes 2 and 5) and experimental (Lanes 3, 4, 6, 7 and 8) Cheddar cheeses after 8 months of ripening. Lane 1 contains a sodium-caseinate standard.

The molecular weight distribution of peptides in water-soluble extracts from the cheeses (as measured by size-exclusion HPLC) serves as a further indication of the extent of proteolysis in the cheeses during ripening; the greater the extent of proteolysis, the higher the level of low molecular weight peptides generated. After 6 months of ripening, the levels of these low molecular weight peptides (< 500 Da) were found to have accumulated to high levels in all cheeses (data not

shown). Moreover, similar levels were detected in the control and experimental cheeses, even in those cheeses which had high levels of survival of adjunct lactobacilli (Trial 1 Vat 3, Trial 2 Vat 4 cheeses), indicating that the extent of proteolysis in the cheeses as demonstrated by generation of small peptides, was not affected by adjunct addition. However higher levels of individual FAA were detected in the cheeses made with added lactobacilli, after 6 months of ripening (Fig. 6).

Fig. 6 depicts the concentration of individual free amino acids in water-soluble extracts of 6 month old control and experimental Cheddar cheeses of Trial 1 (A) and Trial 2 (B).

Most notably, concentrations of serine, methionine, leucine and phenlyalanine (Trial 1) in addition to glutamic acid and valine (Trial 2) were higher in the cheeses made with added lactobacilli than in the control cheese to which no adjunct had been added (Fig. 6). This was found to be true even for the cheeses in which the *Lactobacillus* adjuncts declined during ripening. This may be accounted for by the release of intracellular peptidases as the organisms died and lysed. Thus, in general, the results suggest that the adjunct lactobacilli, whether they survived to high levels or not, did contribute to proteolysis in the cheese as demonstrated by increased formation of FAA.

The above results demonstrate that probiotic *L. paracasei* strains, incorporated into Cheddar cheese proved particularly suitable as starter adjuncts. These strains were found to grow and proliferate to high cell numbers in the cheese over 8 months of ripening, even when added at a relatively low inoculum. Furthermore, RAPD PCR proved extremely useful to distinguish these probiotic adjuncts from NSLAB. Moreover, the results from the control cheese suggest the predominance of certain NSLAB strains. While proteolysis during cheese ripening was influenced by the adjuncts at the level of FAA formation, cheese flavour, texture and appearance were not affected. Incorporation of these probiotic adjuncts into Cheddar cheese, as described herein can be achieved without alteration of the cheesemaking technology, thus

making this system attractive for commercial exploitation. These results indicate that Cheddar cheese is an effective vehicle for delivery of these strains to the consumer with the attendant advantages.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

National Food Biotechnology Centre University College Cork Ireland NAME AND ADDRESS OF DEPOSITOR	**INTERNATIONAL FORM** **RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT** issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Lactobacillus paracaseii NFBC 338	NCIMB 40954

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☒ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 29 May, 1998 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB LTd.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):
Address: 23 St Machar Drive. Aberdeen AB24 3RY	Date: 3 June, 1998

1 Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

National Food Biotechnology Centre
University College
Cork
Ireland

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: As Above Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40954 Date of the deposit or of the transfer[1]: 29 May, 1998

III. VIABILITY STATEMENT
The viability of the microorganism identified under II above was tested on 29 May, 1998 [2]. On that date, the said microorganism was: ☒ viable [3] ☐ no longer viable [3]

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

IV.	CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V.	INTERNATIONAL DEPOSITARY AUTHORITY

Name:	NCIMB Ltd.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):
Address:	23 St Machar Drive Aberdeen AB24 3RY	Date: June 3, 1998

4 Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

National Food Biotechnology Centre
University College
Cork
Ireland

NAME AND ADDRESS OF DEPOSITOR

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Lactobacillus paracaseii NFBC 364	NCIMB 40955

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☒ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 29 May, 1998 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB LTd.

Address: 23 St Machar Drive,
Aberdeen
AB24 3RY

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):

Date: 3 June 1998

1 Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.
Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

National Food Biotechnology Centre University College Cork Ireland	**INTERNATIONAL FORM** **VIABILITY STATEMENT** **issued pursuant to Rule 10.2 by the** **INTERNATIONAL DEPOSITARY AUTHORITY** **identified on the following page**

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: As Above Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40955 Date of the deposit or of the transfer[1]: 29 May, 1998

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 29 May, 1998 [2]. On that date, the said microorganism was:

☒ [3] viable

☐ [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: NCIMB Ltd. Address: 23 St Machar Drive Aberdeen AB24 3RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s): Date: June 3, 1998

4 Fill in if the information has been requested and if the results of the test were negative.

CLAIMS:

1. A process for the manufacture of a probiotic cheese, which process comprises adding a 0.05-0.5% inoculum of a strain of *Lactobacillus paracasei*, which is non-pathogenic, acid and bile tolerant and adherent to human epithelial cells, as a starter adjunct to cheese milk, said *L. paracasei* strain being capable of growing during the ripening phase to a level of 10^7cfu/g or greater.

2. A process according to Claim 1, wherein a 0.1-0.25% inoculum of the *L. paracasei* is added to the cheese milk.

3. A process according to Claim 1 or 2, wherein the ripening phase is at least six months.

4. A process according to any preceding claim, wherein the ripening phase is eight months or greater.

5. A process according to any preceding claim, wherein the *L. paracasei* is capable of growing during the ripening phase to a level of 10^8cfu/g or greater.

6. A process according to any preceding claim, wherein the *L. paracasei* is tolerant to temperatures of 37°C or greater.

7. A process according to any preceding claim, wherein the *L. paracasei* can be enumerated and distinguished from the resident flora.

8. A process according to Claim 7, wherein the added *L. paracasei* cells are enumerated and distinguished by a randomly amplified polymorphic DNA (RAPD) method which allows the generation of discrete DNA fingerprints for the respective strains.

9. A process according to any preceding claim, wherein the cheese manufactured is a hard cheese.

10. A process according to Claim 9, wherein the cheese is Cheddar cheese.

11. A process according to Claim 1, substantially as hereinbefore described and exemplified.

12. *Lactobacillus paracasei* strain NFBC 338 or a mutant or variant thereof.

13. *Lactobacillus paracasei* strain NFBC 364 or a mutant or variant thereof.

14. A probiotic cheese ready for consumption which contains a viable, actively growing strain of *L. paracasei* as defined in any one of Claims 1-13 in an amount of 10^7cfu/g or greater, following manufacture thereof using said *L. paracasei* as a starter adjunct.

15. A probiotic cheese according to Claim 14, which is Cheddar cheese.

16. A probiotic cheese according to Claim 14, substantially as hereinbefore described and exemplified.

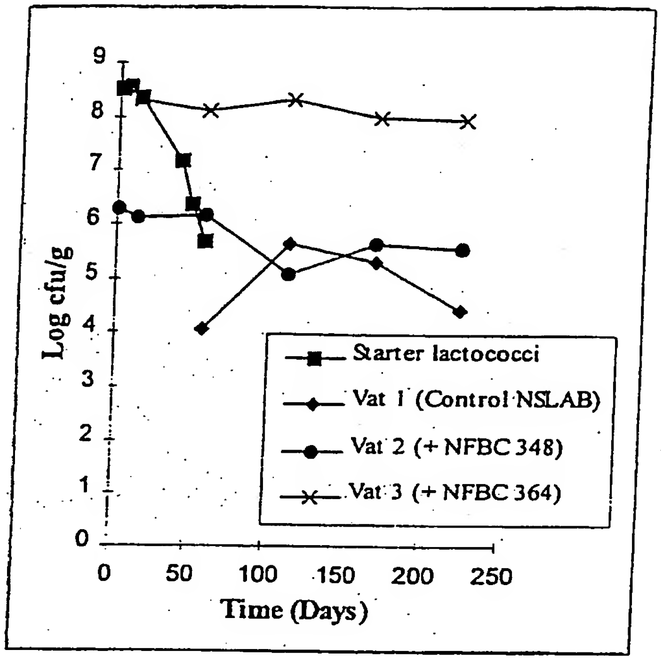
Log cfu/g
Time (Days)
0
1
2
3
4
5
6
7
8
9
0
50
100
150
200
250
Starter lactococci
Vat 1 (Control NSLAB)
Vat 2 (+ NFBC 348)
Vat 3 (+ NFBC 364)

Fig. 1A

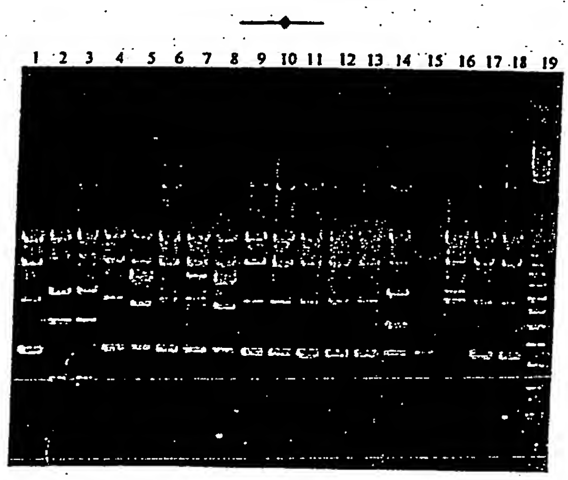
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Fig. 1B

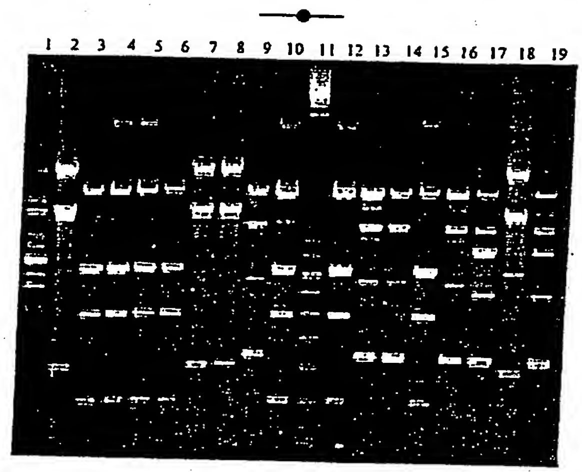
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig. 1C

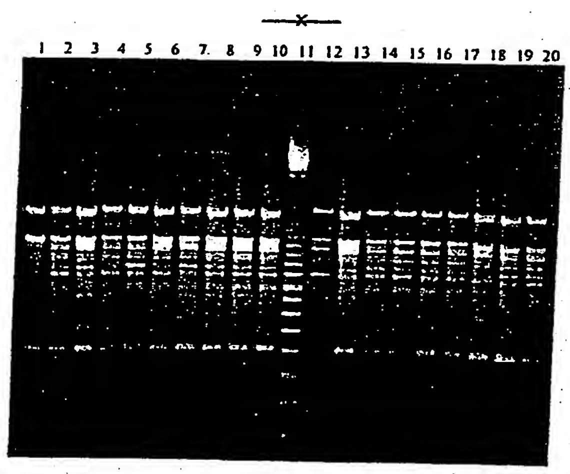
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fig. 1D

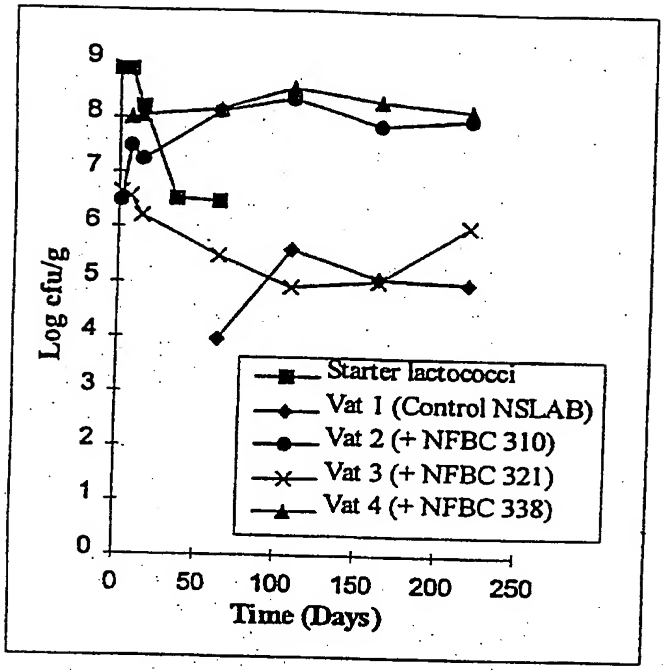
9
8
7
6
5
4
3
2
1
0
Log cfu/g
0
50
100
150
200
250
Time (Days)
Starter lactococci
Vat 1 (Control NSLAB)
Vat 2 (+ NFBC 310)
Vat 3 (+ NFBC 321)
Vat 4 (+ NFBC 338)

Fig. 2A

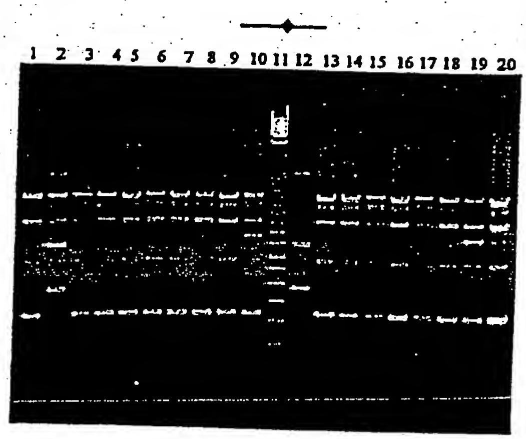
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Fig. 2B

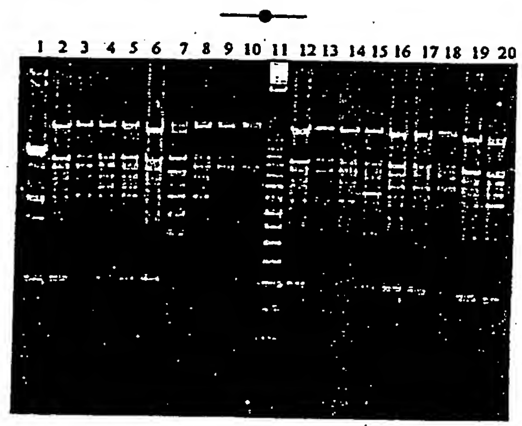

Fig. 2C

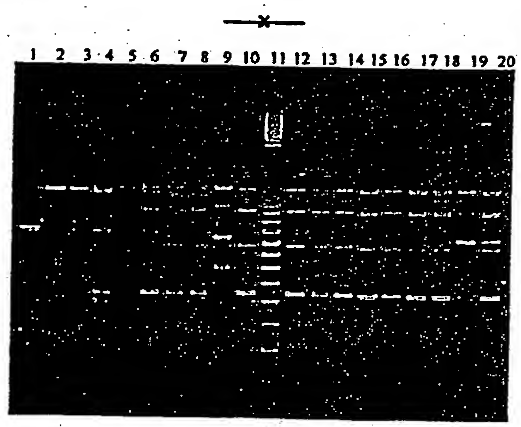

Fig. 2D

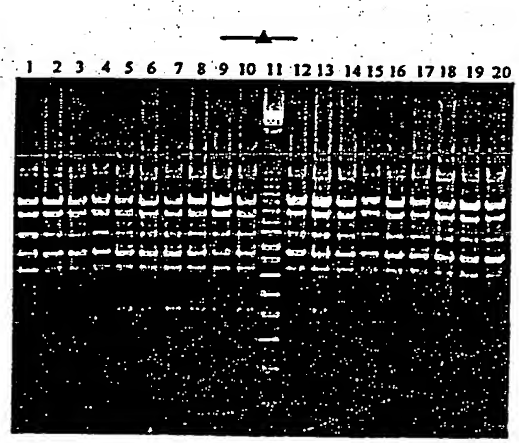

Fig. 2E

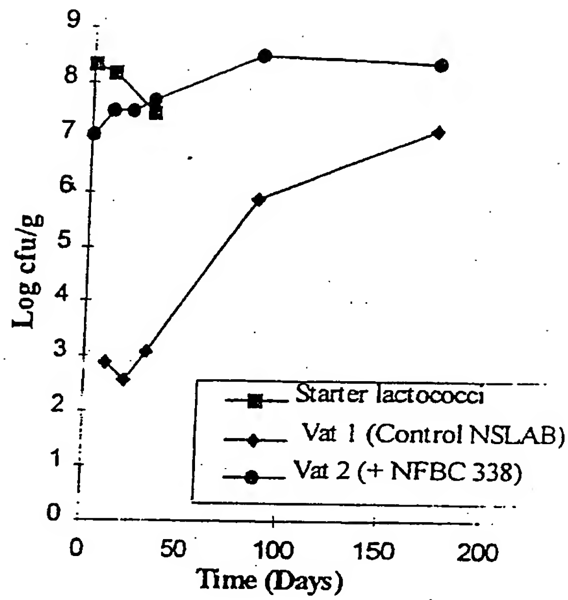
9
8
7
6
5
4
3
2
1
0
Log cfu/g
0
50
100
150
200
Time (Days)
Starter lactococci
Vat 1 (Control NSLAB)
Vat 2 (+ NFBC 338)

Fig. 3A

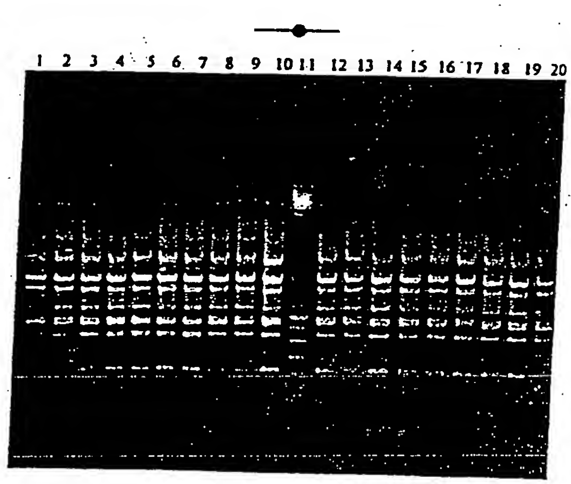
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Fig. 3B

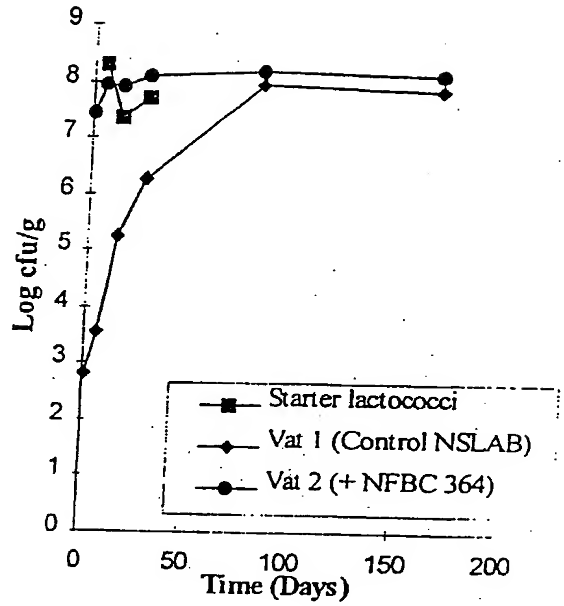
9
8
7
6
5
4
3
2
1
0
Log cfu/g
0
50
100
150
200
Time (Days)
Starter lactococci
Vat 1 (Control NSLAB)
Vat 2 (+ NFBC 364)

Fig. 4A

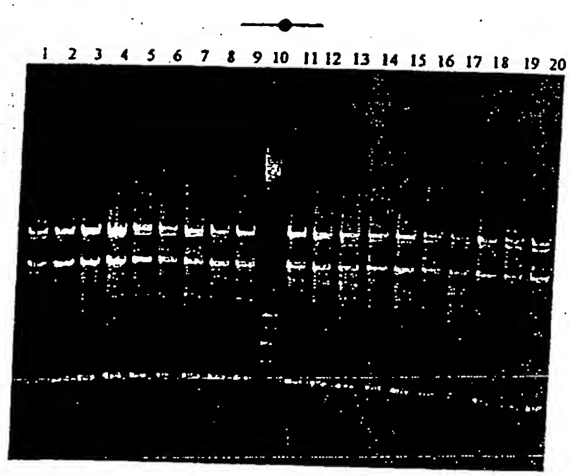
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Fig. 4B

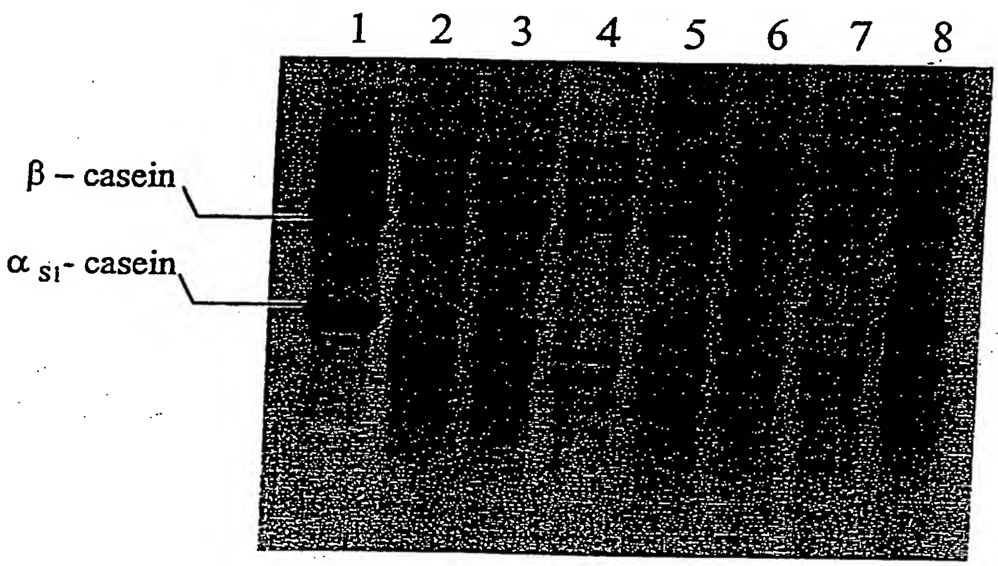

Fig. 5

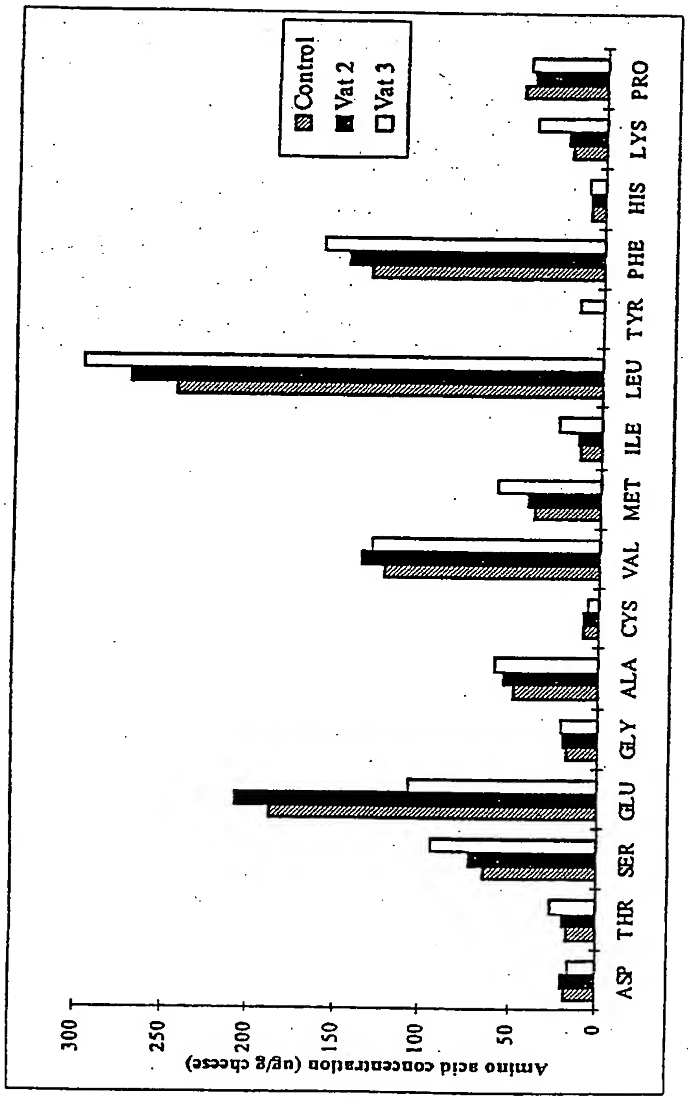
Control
Vat 2
Vat 3
Amino acid concentration (ug/g cheese)
0
50
100
150
200
250
300
ASP
THR
SER
GLU
GLY
ALA
CYS
VAL
MET
ILE
LEU
TYR
PHE
HIS
LYS
PRO

Fig. 6A

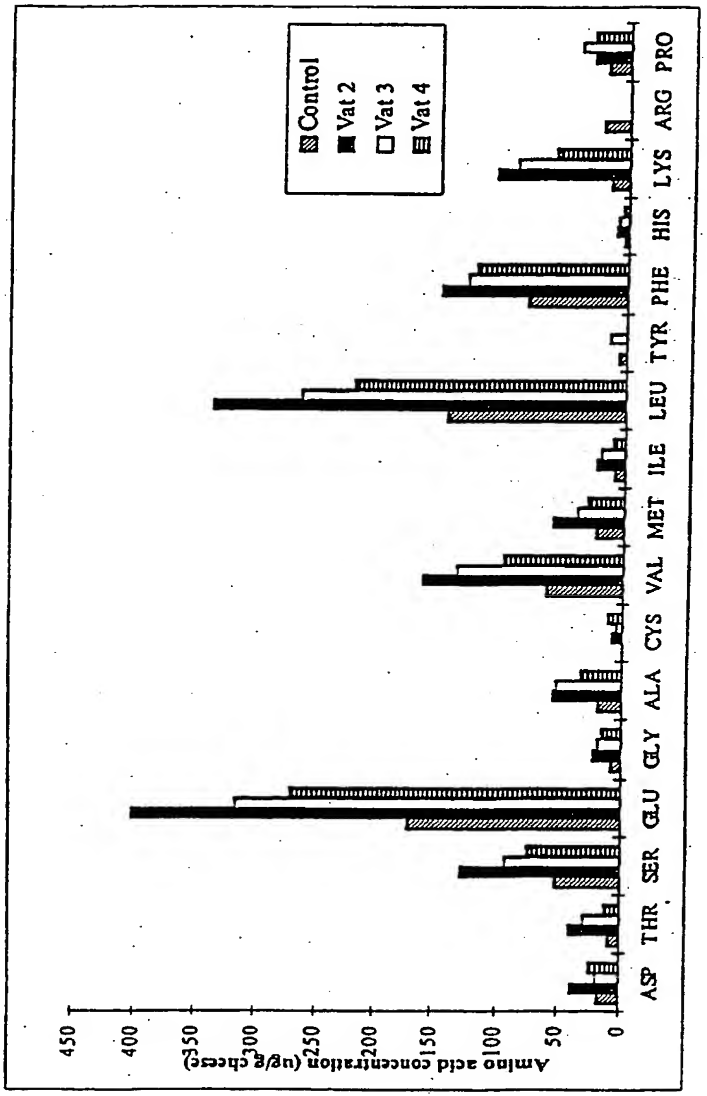
Control
Vat 2
Vat 3
Vat 4
Amino acid concentration (ug/g cheese)
0
50
100
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Fig. 6B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IE 99/00047

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A23C19/032 C12R1/245

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A23C C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P, O	T. KLAENHAMMER: "Probiotic cheese" INTERNATIONAL DAIRY JOURNAL , vol. 8, no. 5/6, 1998, pages 491-496, XP002116067 page 491 -page 493; figure 2 ---	1-16
L	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANFURT/MAIN, DE AN= 1999-00-p1445, C. STANTON: "Probiotic cheese" XP002115138 abstract & INTERNATIONAL DAIRY JOURNAL , vol. 8, no. 5/6, 1998, pages 491-496, This paper is from a symposium held in Cork on 30 Sept. to 2 Oct. 1997 ---	

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 1999	13/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Desmedt, G

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INTERNATIONAL SEARCH REPORT

Interne 'al Application No
PCT/IE 99/00047

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	C. LYNCH: "Manufacture of cheddar cheese with and without adjunct Lactobacilli under controlled microbiological conditions" INTERNATIONAL DAIRY JOURNAL, vol. 6, no. 8, 1966, pages 851-867, XP002115824 page 851; figures 1,6; table 3 page 856	1-7, 9-11, 14-16
X	GOMEZ M J ET AL: "DEBITTERING ACITIVITY OF PEPTIDASES FROM SELECTED LACTOBACILLI STRAINS IN MODEL CHEESES" MILCHWISSENSCHAFT, vol. 51, no. 6, 1 January 1996 (1996-01-01), pages 315-319, XP000624592 ISSN: 0026-3788 page 316; table 4	1,2,5-7, 9-11, 14-16
X	LEE B H ET AL: "INFLUENCE OF HOMOFERMENTATIVE LACTOBACILLI ON THE MICROFLORA AND SOLUBLE NITROGEN COMPONENTS IN CHEDDAR CHEESE" JOURNAL OF FOOD SCIENCE, vol. 55, no. 2, 1 March 1990 (1990-03-01), pages 391-397, XP000126289 ISSN: 0022-1147 page 391 -page 392; figures 1-4	1-10, 14-16
X	LEE B H ET AL: "INFLUENCE OF HOMOFERMENTATIVE LACTOBACILLI ON PHYSICOCHEMICAL AND SENSORY PROPERTIES OF CHEDDAR CHEESE" JOURNAL OF FOOD SCIENCE, vol. 55, no. 2, 1 March 1990 (1990-03-01), pages 386-390, XP000126288 ISSN: 0022-1147 page 386, column 2 -page 387, column 1; table 1	1-10,14, 16
X	R. HARGROVE: "New type of ripened low-fat cheese" JOURNAL OF DAIRY SCIENCE, vol. 49, no. 7, 1966, pages 796-799, XP002115136 CHAPAIGN, ILLINOIS US page 797	1,2,6, 9-11,14, 15

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BROOME M C ET AL: "THE USE OF NON-STARTER LACTOBACILLI IN CHEDDAR CHEESE MANUFACTURE" AUSTRALIAN JOURNAL OF DAIRY TECHNOLOGY, vol. 45, no. 2, 1 November 1990 (1990-11-01), pages 67-73, XP000166076 ISSN: 0004-9433 page 67, column 2 -page 68, column 1 ---	1,3-7, 9-11, 14-16
A	E. TUOMOLA: "ADDITION OF SOME PROBIOTIC AND DAIRY LACTOBACILLUS STRAINS TO CACO-2 CELL CULTURES" INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, vol. 41, no. 1, 1998, pages 45-51, XP002115137 page 45 -page 46; table 1 ---	1
L	FOX P F ET AL: "SIGNIFICANCE OF NON-STARTER LACTIC ACID BACTERIA IN CHEDDAR CHEESE" AUSTRALIAN JOURNAL OF DAIRY TECHNOLOGY, vol. 53, no. 2, 1 June 1998 (1998-06-01), pages 83-89, XP000772490 ISSN: 0004-9433 Lb. paracasei = formerly Lb. casei subsp. pseudoplantarum. page 83, column 2 -----	